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Botryococcus braunii strains compared for biomass productivity, hydrocarbon and carbohydrate content

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ABSTRACT

Botryococcus braunii can produce both long-chain hydrocarbons as well as carbohydrates in large quantities, and is therefore a promising industrial organism for the production of biopolymer building blocks. Many studies describe the use of different strains of *Botryococcus braunii* but differences in handling and cultivation conditions make the comparison between strains difficult. In this study, 16 *B. braunii* strains obtained from six culture collections were compared for their biomass productivity and hydrocarbon and carbohydrate content. Biomass productivity was highest for AC768 strain with 1.8 g L^{-1} day^{-1}, while hydrocarbon production ranged from none to up to 42% per gram biomass dry weight, with Showa showing the highest hydrocarbon content followed by AC761. The total carbohydrate content varied from 20% to 76% per gram of the biomass dry weight, with CCALA777 as the highest producer. Glucose and galactose are the main monosaccharides in most strains and fucose content reached 463 mg L^{-1} in CCALA778.

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1. Introduction

Human activities greatly depend on petroleum as both an energy source and industrial raw material (Dale, 2007). Petroleum usage in the long term is both unsustainable due to depleting economically relevant sources and by rapid release of carbon dioxide in the environment. One potential source of biofuels and other biobased raw material compounds are microalgae. These photoautotrophic organisms are able to transform inorganic carbon into lipids such as triacylglycerols (TAGs) at a faster rate than agricultural oleaginous crops, and do not compete for arable land (Wijffels and Barbosa, 2010). Besides lipids, other products of interest that microalgae may produce in large quantities are hydrocarbons and carbohydrate. Hydrocarbons are natural occurring compounds consisting entirely of hydrogen and carbon, and are one of the most important energy resources (Timmis and Qin, 2010). Hydrocarbons derived from microalgae can be hydrocracked and transformed into aviation turbine fuel (Hillen et al., 1982). Carbohydrate have a range of industrial uses, including as thickeners, stabilisers and gelling agents in food products (Donot et al., 2012), as well as in the pharmaceutical and cosmeceutical industries (Borowitzka, 2013; Buono et al., 2012).

One promising production host of biofuels and biobased materials is *Botryococcus braunii*. This eukaryotic microalga specie can be found across the world as a variety of strains with different physiological characteristics. Some strains of *B. braunii* can produce to up to 86% hydrocarbons on cell dry weight basis (Brown et al., 1969), whereas other strains can produce to up to 4.5 g L^{-1} carbohydrates into the medium (Fernandes et al., 1989). One advantage of *B. braunii* is that it secrets extracellular hydrocarbons and carbohydrates (Kalacheva et al., 2002; Lupi et al., 1994; Volova et al., 1998; Weiss et al., 2012; Wolf, 1983) which allows the development of strategies for *in situ* extraction such as "milking" (Moheimani et al., 2013).

B. braunii can produce hydrocarbons with different chemical structures. These hydrocarbons play a role in the natural growth cycle of *B. braunii* (Khatri et al., 2014). Depending on what type

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Table 1
Origen of *Botryococcus braunii* strains. Information regarding the individual strains and the culture collections from where they were purchased. * strains used for growth in bubble columns photobioreactors.

Culture collection	*Botryococcus braunii* Strain		Race	Location	Isolation, date of isolation	Reference
Berkeley	Showa	*	Race B	culturing tanks, Berkley	By unknown, 1980	Wolf et al. (1985)
Scandinavian Culture Collection of Algae and Protozoa (SCCAP)	SCCAP K-1489		Not know	Belgium, Nieuwoort	By G. Hansen, 2008	No reference
Culture Collection of Algae at Goettingen University (SAG)	SAG 30.81		Race A	Peru, Dpto. Cuzco, Laguna Huaypo	By E. Hegewald, 1977	Dayananda et al. (2007)
Culture Collection of Autotrophic Organisms (CCALA)	CCALA-777	*	Not know	Porto da Castanheira (Poço dos Basilios) Portugal	By Santos, 1975	Fernandes et al. (1989)
	CCALA-778	*	Not know	Serra da Estrela (Barragem da Erva da Fome) Portugal	By Santos, 1997	No reference
	CCALA-835		Race A	Peru, Dpto. Cuzco, Laguna Huaypo	By E. Hegewald, 1977	Dayananda et al. (2007)
Culture Collection of Algae and Protozoa (CCAP)	CCAP-807-2	*	Race A	Grasmere, Cumbria, England	By Jaworski, 1984	Hilton et al. (1989)
UTEX Culture Collection of Algae	UTEX Bb 572		Race A	Madingley, Cambridge, England	By M.R. Droop, 1950	Eroglu and Melis (2010)
	UTEX Bb LB572		Race A	Cambridge, England	By M.R. Droop, 1950	Eroglu et al. (2011)
ALGOBANK-CANE	AC755	*	Race A	Lingoult-Morvan, France	By Pierre Metzger, 1981	Metzger et al. (1985a,b)
	AC759	*	Race B	Ayame, Ivory Coast	By Pierre Metzger, 1984	Metzger et al. (1988)
	AC760		Race B	Kossou, Ivory Coast	By Pierre Metzger, 1984	Metzger et al. (1988)
	AC761	*	Race B	Paquemar, Martinique, France	By Pierre Metzger, 1983	Metzger et al. (1985a,b)
	AC765		Race L	Kossou, Ivory Coast	By Pierre Metzger, 1984	Metzger et al. (1988)
	AC767		Race L	Songkla Nakarin, Thailand	By Pierre Metzger, 1985	Metzger et al. (1987)
	AC768		Race L	Yamoussoukro, Ivory Coast	By Pierre Metzger, 1984	Metzger et al. (1987)

of hydrocarbons are produced, *B. braunii* is subclassified into four chemical races, designated A, B and L (Metzger and Largeau, 2005), and S, a recent assignment (Kawachi et al., 2012). Race A strains synthesize odd-numbered alkadienes and trienes (C_{25} to C_{31}) (Dayananda et al., 2007; Eroglu and Melis, 2010; Hilton et al., 1988; Metzger et al., 1986; Metzger et al., 1989), race B strains synthesize a class of isoprenoid derived compounds termed botryococcenes (C_{30} to C_{37}) and methylated squalenes (C_{31} to C_{34}) (Metzger and Casadevall, 1983; Metzger et al., 1985b; Metzger et al., 1987; Nanamura, 1988), race L strains synthesize lycopadiene (C_{40}) and race S strains synthesize C_{18} epoxy-*n*-alkanes and C_{20} saturated *n*-alkanes (Metzger and Casadevall, 1987).

In addition to hydrocarbons, *B. braunii* strains can produce large amounts of carbohydrates with the highest amounts so far reported as 4.0–4.5 $g\,L^{-1}$ (Fernandes et al., 1989). Extensive carbohydrate production was first observed by an increase of broth viscosity during growth (Casadevall et al., 1985). Since this initial report, other strains were found to produce carbohydrates with yields of 250 $mg\,L^{-1}$ for race A and B strains, and 1 $g\,L^{-1}$ for a race L strain (Allard and Casadevall, 1990). Later, 1.6 $g\,L^{-1}$ for *B. braunii* LB572 and 0.7 $g\,L^{-1}$ for SAG30.81 (Dayananda et al., 2007) were reported. Galactose was identified as the main monomeric sugar constituent of all carbohydrates examined, with fucose and rhamnose as accompanying monomers. Glucose was detected in the L strain only (Allard and Casadevall, 1990).

One drawback of using *B. braunii* as an industrial host is its slow growth compared to other photoautotrophic microorganisms. *B. braunii* biomass productivities range between 0.1 and 0.2 $g\,L^{-1}\,d^{-1}$ (Cabanelas et al., 2015; Eroglu et al., 2011) where other green microalgae such as *Chlorella* sp. can achieve 0.5 $g\,L^{-1}\,d^{-1}$ (Hempel et al., 2012). One commonly reported hypothesis for the slow growth is due to the synthesis of energetically expensive hydrocarbons (Banerjee et al., 2002).

There is an extensive body of work in last few decades describing different strains of *B. braunii* and it is clear that there is a high degree of morphological plasticity and physiological diversity amount the genus. It is probably due to this high diversity in the genus that *B. braunii* is not an easy organism to maintain and grow under laboratory conditions. Many methods of cultivation, different types of growth medium or culture conditions have been reported in the literature to study more in depth *B. braunii* individual strains as well as for comparing strains diversity (Allard and Casadevall, 1990;

Table 2
Hydrocarbon profile of seven strains of *Botryococcus braunii* grown in shake flasks. Strains are grouped into subclades 3 and 5 as a result of the phylogenetic placement results. Superscript letter *a* and *b* next to chemical formulas stand for different molecular structures. C? refers to unidentified compound.

Chemical formula	Subclade 3				Subclade 5		
	Showa	AC759	AC760	AC761	K-1489	AC755	CCAP807/2
$C_{23}H_{44}$_320	–	–	–	-	–	–	73
$C_{25}H_{48}$_348	–	–	-	–	–	48	93
$C_{27}H_{52}$_376	–	–	–	–	–	58	171
$C_{29}H_{56}$_404	–	–	–	–	97	283	187
$C_{31}H_{60}$_432	–	–	–	–	65	94	–
$C_{33}H_{56}$_452[a]	–	55	58	–	–	–	–
$C_{33}H_{56}$_452[a]	91	–	–	109	–	–	–
$C_{34}H_{58}$_466[b]	108	–	–	57	–	–	–
$C_{34}H_{58}$_466[b]	599	–	57	509	–	–	–
$C_{34}H_{58}$_466[b]	138	195	334	186	–	–	–
$C_{34}H_{58}$_466[b]	84	270	240	77	–	–	–
C?	133	–	–	–	–	–	–
total hydrocarbons (mg L^{-1})	1153	520	689	938	162	483	524

Cabanelas et al., 2015; Dayananda et al., 2007; Eroglu et al., 2011; Hegedűs et al., 2016; Metzger et al., 1989; Moutel et al., in press). While this has led to several reports on hydrocarbon and carbohydrate production for various *B. braunii* strains, it can be argued that it is still difficult to compare and assess physiological characteristics. This study attempts to establish reference conditions for future investigations of this interesting photosynthetic organism alongside its production of long chain hydrocarbons and/or carbohydrates.

The aim was to compare various readily available strains of *B. braunii* under the same culture conditions for biomass productivity and total hydrocarbon and carbohydrate content. Sixteen strains were tested in Erlenmeyer flasks, of which seven promising strains were additionally investigated in bubble columns. Comparison of the sixteen strains regarding biomass productivity, carbohydrate and hydrocarbon content is reported and major differences highlighted. Two strains are put forward as candidates for industrial applications.

2. Materials and methods

2.1. Strains and media

Sixteen non-axenic *B. braunii* strains were obtained from culture collections (Table 1). Upon arrival, each strain was washed with sterile distilled water and revegetated in modified Chu 13 medium (Largeau et al., 1980) without citric acid or vitamins, with the following composition: 400 mg L^{-1} KNO_3, 200 mg L^{-1} $MgSO_4 \cdot 2H_2O$, 108 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 104.8 mg L^{-1} K_2HPO_4, 20 mg L^{-1} Fe–Na_2EDTA, 9.4 μg L^{-1} Na_2O_4Se, 2.86 mg L^{-1} H_3BO_3, 1.8 mg L^{-1} $MnSO_4 \cdot 4H_2O$, 220 μg L^{-1} $ZnSO_4 \cdot 7H_2O$, 90 μg L^{-1} $CoSO_4 \cdot 7H_2O$, 80 μg L^{-1} $CuSO_4 \cdot 5H_2O$, 60 μg L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 10 μL L^{-1} H_2SO_4. Final pH was adjusted to pH 7.2 with NaOH. The 16 strains were kept in an incubation room with the initial environment parameters: light intensity of 60 μmol photon m^{-2} s^{-1}, light:dark photoperiod 18:6 h, ambient air CO_2, temperature of 23 °C and mechanical shaking at 60 rpm. Growth conditions – prior to the experiment, the 16 strains inoculum were cultivated for two batch cycles under experimental conditions to minimize effects of changing environmental parameters.

Shake flask cultivation: For comparison of biomass productivity, hydrocarbon and carbohydrate content, the 16 strains were grown in Infors HT Multriton incubators in 250 mL conical flasks and a volume of 150 mL medium. Temperature was set at 23 °C, with 2.5% CO_2 enriched air and shaking at 90 rpm. Illumination was provided by Phillips lamps FL-Tube L 36W/77, with 150 μmol photon m^{-2} s^{-1}, and a light:dark photoperiod of 18:6 h (photoperiod of 18:6 h chosen based on earlier work done as described in Gouveia, 2010). Flasks were inoculated with algae from a 10-day old, actively growing culture, such that the initial absorbance at 680 nm was 0.2. The Erlenmeyer flasks were capped with aeraseal sterile film (Alphalabs). The experiment was conducted in triplicate and samples were taken at days 0, 6, 12 and 18 after inoculation. The cultivation period of 18 days for this experiment, was chosen based on similar cultivation times found in other studies (Eroglu and Melis, 2010; Kojima and Zhang, 1999).

Bubble columns cultivation: For determining cultivation viability at larger scale of selected strains from shake flask experiment, seven strains (marked with a star in Table 1) were cultivated in batch mode in a bubble column with a working volume of 400 mL. These custom-made glass tubes (Glass Instrumentmakerij, Wageningen University) are 400 mm in height, with an internal and external diameter of 40 and 60 mm, respectively. A water bath was used to pump water through an external water jacket, keeping the temperature of the cultures at 23 °C. The bubble columns were kept vertical between two large light panels (bench panels: Mazda 5L KN RB ECO 118 I, fluorescent tubes: Philips Master TL-D 18W/865) which provided 150 μmol photon m^{-2} s^{-1} from two sides with a light:dark cycle of 18:6 h. Air enriched with 2% CO_2 was continuously injected at the bottom of the reactor at a rate of 0.5 vvm (volume air volume $reactor^{-1}$ min^{-1}, 200 mL min^{-1}). Air flow was controlled by a mass flow controller (Brooks 0254, Brooks instruments), and filter sterilized through a 0.2 μm air filter (Acro 50, Pall Corporation) before it passed through a holed plate in the tube to create small bubbles. The enriched air provided inorganic carbon for growth, kept the reactor mixed and stabilized pH around 7.2. To compensate for evaporation, Milli Q water was added to the aeration tubes through a 0.2 μm filter (Minisart, Sartorius stedim). An overflow bottle equipped with 0.2 μm air filter (Acro 50, Pall Corporation) was connected to the top of the column, to enable air to escape and possible overflow of the culture to be kept free of contamination. The reactors were inoculated at biomass concentration of 0.1–0.3 g L^{-1}, and sterile medium was added to 400 mL. Twice a week samples were taken through a port at the side of the reactor to determine pH and dry weight.

2.2. Biomass dry weight

Five mL aliquots of culture broth were filtered onto pre-weighed GF/D glass-fibre membranes (Whatman). The GF/D filters were dried at 100 °C for 24 h and weighted, and biomass amount was determined by subtraction. From the dry weight, biomass productivity was calculated using the following equation:

$$\text{Productivity} = \frac{C_{x_2} - C_{x_1}}{t_2 - t_1},$$

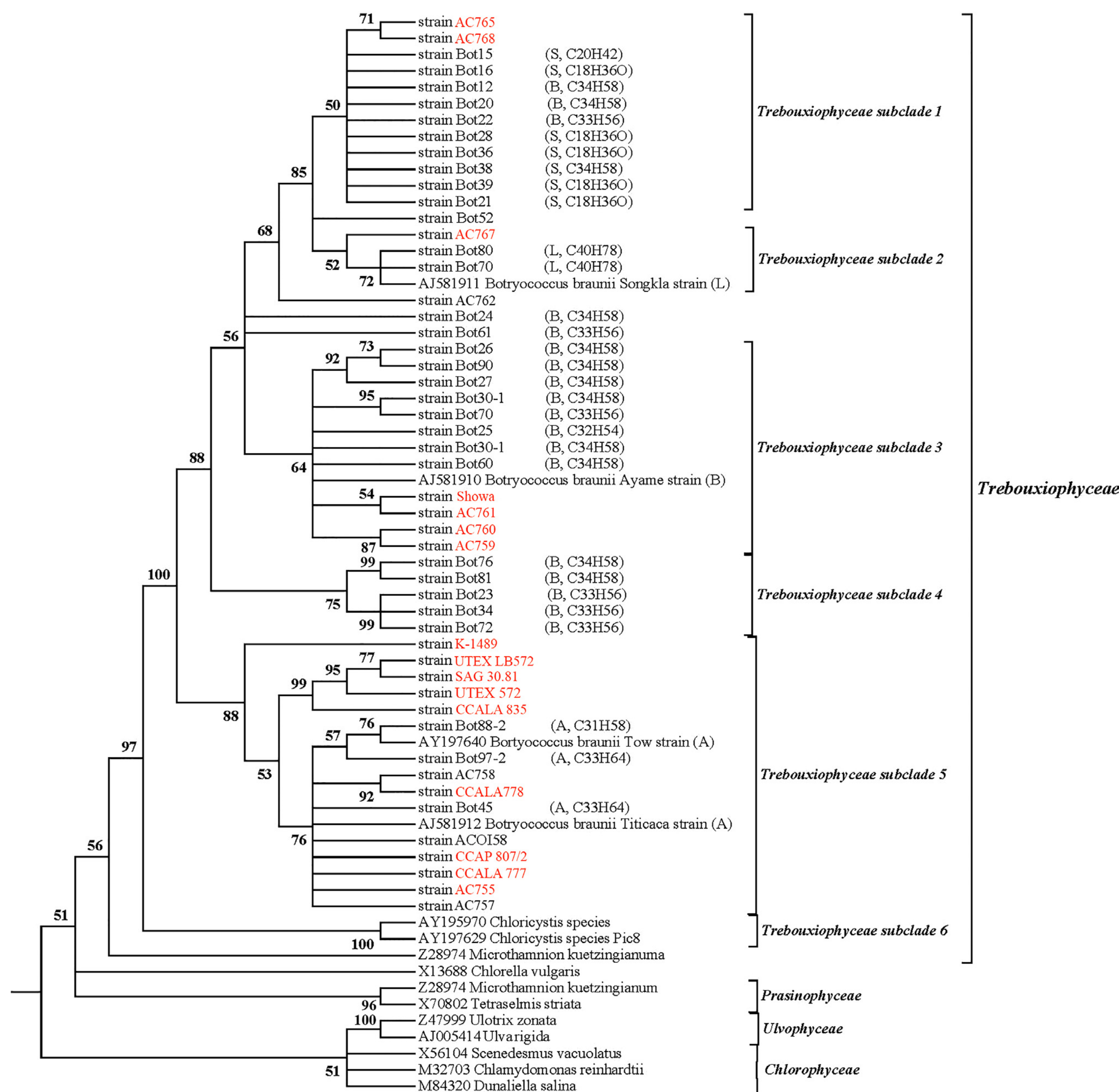

Fig. 1. Phylogeny tree placement of 16 *Botryococcus braunii* strains. Strict consensus tree based with overlaid bootstrap values and concensus threshold of 50% obtained by maximum-likelihood analysis on 18-rRNA gene sequences from 19 *Botryococcus* culture collection strains, 4 reference strains AJ581910 Ayame (B), AJ581911 Songkla (L), AJ581912 (A) and AY197640 Tow (A), 31 Bot strains from Kawachi et al., 2012 and species from other chlorophytes that are used as an outgroup.

where C_{x_1} and C_{x_2} represent the biomass concentration at the beginning (t_1) and at the end of the experiment (t_2), respectively. A univariate analysis of variance (one-way ANOVA) was carried out to compare the biomass productivity among different strains. As post-hoc test the Tukey's test was used. All statistical analyses were done at a significance level of 0.05. The software used was Genstat 64-bit Release 18.1.

2.3. Hydrocarbon extraction

Our method is adapted from the methodology developed by Folch and Dyer (Bligh and Dyer, 1959; Folch et al., 1957). One milliliter of culture was transferred to a glass vial and 2.5 mL methanol and 1.25 mL dichloromethane were added and mixed for 6 h. After the mixing step, 1.25 mL dichloromethane was added and mixed for 1 min followed by addition of 1.25 mL 0.9% (w/v) NaCl and mixed for another minute. Hereafter, samples were centrifuged for 5 min at 1500 × *g* and the bottom phase was removed to a new glass vial using a glass Pasteur pipette and dried under nitrogen gas. The residue was resuspended in 300 μL dichloromethane:methanol (v:v) and stored at −20 °C. For the hydrocarbon extraction of the strains cultivated in the bubble columns, the residue was resuspended in 1000 μL hexane. Hydrocarbon extraction was performed using the last data point samples for both the Erlenmeyer flasks and bubble column cultures.

2.4. Hydrocarbon analysis

Hydrocarbons extracted from the strains cultivated in the Erlenmeyer flasks were measured by gas chromatography com-

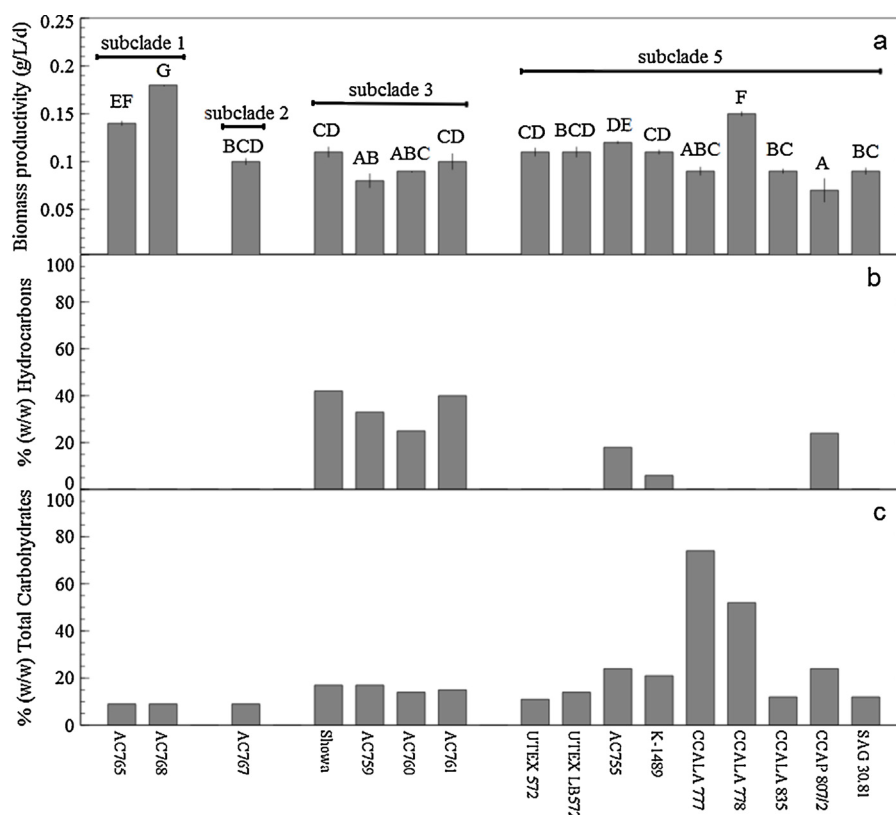

Fig. 2. Comparison of physiological traits for 16 *Botryococcus braunii* strains. (a) Biomass productivity; (b) total hydrocarbon content; (c) total carbohydrate content. The bars in plot A represent the standard error mean, with 3 replicates for all except AC760 and AC768 which 1 replicate was used, and AC755, AC761, AC767, CCAP807/2, SAG30.81, CCALA778, UTEX LB572 where 2 were used. Each letters above bars in graph a, represent statistical difference ($P < 0.05$) by ANOVA.

Table 3
Monosaccharide composition. Monomeric sugar content (mg L^{-1}) of the 16 strains of *Botryococcus braunii* grouped by subclades from the phylogenetic placement results. The entry n.d. stands for not detected.

	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Total
Subclade 1						
AC765	37	3	27	99	118	284
AC768	70	3	12	131	113	328
Subclade 2						
AC767	24	2	17	144	21	208
Subclade 3						
Showa	6	3	102	199	163	473
AC759	n.d	5	18	210	82	316
AC760	n.d	4	18	165	99	286
AC761	n.d	3	17	230	93	343
Subclade 5						
UTEX 572	4	3	10	107	158	282
UTEX LB572	7	3	9	86	213	319
AC755	172	3	27	307	151	660
K-1489	80	3	9	321	163	576
CCALA 777	279	4	9	1115	374	1781
CCALA 778	463	9	21	908	288	1688
CCALA 835	29	3	14	88	143	277
SAG 30.81	18	9	15	72	123	237
CCAP 807/2	136	5	28	134	178	480

bined with mass spectrometry (GC–MS). The instrument used was a 7890A/5975C from Agilent Technologies, using a DB-Petro (100 m × 0.25 mm × 0.50 μm) J&W Column using helium as the carrier gas, splitless injector, an operation temperature of 280 °C and an injection volume of 1 μL. The oven program was set at 40 °C for 0.5 min, then ramped up by 30 °C per minute to 250 °C followed by a 5 °C per minute ramp to 300 °C in 37.5 min, with a total run time of 55 min. Samples were diluted in dichloromethane:methanol 1:1 (v:v) Squalene ($C_{30}H_{50}$, M = 410 g mol^{-1}) was the reference standard used. The concentration of the calibration standards were 50, 100, 250 and 500 mg L^{-1}.

Hydrocarbon analysis for the strains cultivated in the bubble columns was carried out using GC-FID. The instrument used was an Agilent Technologies HP6890 series equipped with auto sampler, a using Restek Rxi-5ms (30 m × 0.25 mm × 0.25 μm) column. Helium was used as the carrier gas, and a hydrogen/air mixture detection, gas splitless injectors at 350 °C oven temperature and injection volume of 1 μL. The oven program was 50 °C for 1 min, then 15 °C per minute to 180 °C, then 7 °C per minute to 230 °C,

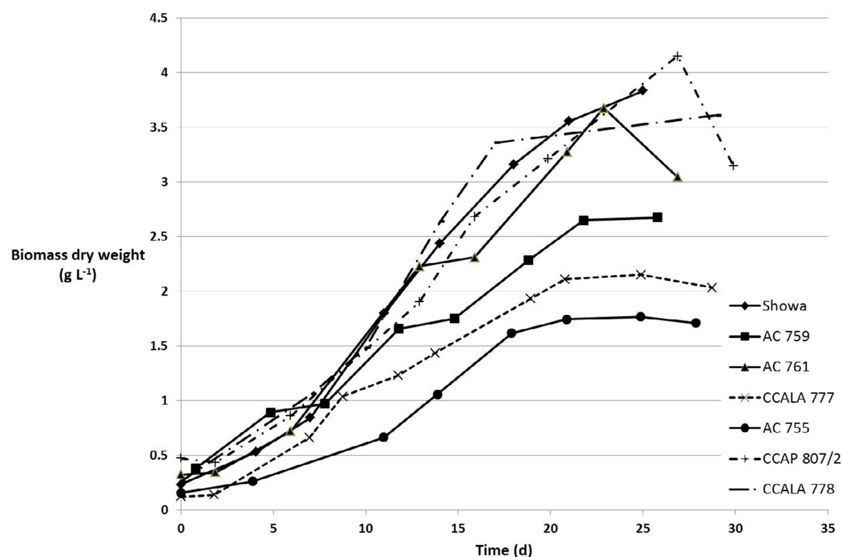

Fig. 3. Growth curves of *Botryococcus braunii* strains in bubble columns photobioreactors. Cultivation in batch mode in the range of 25–30 days. $n = 1$.

then 30 °C per minute to 350 °C and hold for 15 min with a total running time of 35 min. Samples were diluted in hexane, and several dilutions of standards using squalene were used. The dilutions were 1, 0.1, 0.01, 0.005 and 0.001% (v/v).

2.5. Carbohydrate extraction

10 mL of culture from the last time point taken, was freeze dried using Zirbus technology Sublimator 2 × 3 × 3 and the pellet weight was determined. A pre-hydrolysis step was performed as follows: 72% (w/w) H_2SO_4 was added to the freeze dried sample (w/v) and hydrolyzed for 1 hour at 30 °C, and stirred with glass pipette every 15 min. After the pre-hydrolysis water was added in 11.6 v/v (final concentration 1 M H_2SO_4) to the mixture and placed at 100 °C for 3 h and shaken every 30 min. Hereafter the solution was allowed to cool on ice for 15 min and subsequently centrifuged for 15 min at 3000 × *g*. Before analysis samples were diluted in water and 2.5 μL mL^{-1} 0.1% (w/v) bromophenol blue in ethanol was added to the samples. For neutralization the pH, solid barium carbonate powder was added until a magenta colour was seen. Subsequently the solution was filtered through a 0.45 μm pore size PTFE filter.

2.6. Carbohydrate analysis

Monomeric sugar composition was determined by High Performance Anion Exchange Chromatography (HPAEC) using an ICS-3000 Ion Chromatography HPLC system equipped with a CarboPac PA-1 column (2 mm × 250 mm) in combination with a CarboPac PA guard column (2 mm × 25 mm) and a pulsed electrochemical detector in pulsed amperometric detection mode (Dionex). A flow rate of 0.3 mL min^{-1} was used and the column was equilibrated with 16 mM NaOH. The following gradient was used: 0–26 min, 16 mM NaOH; 26–33 min, 16–100 mM NaOH; 33–78 min 0–1 M sodium acetate in 100 mM NaOH; 78–83 min 1 M sodium acetate in 100 mM NaOH. L-Rhamnose, L-fucose, D-mannose, L-arabinose, D-glucose, D-xylose, D-galactose, sucrose, D-glucuronic acid and D-galacturonic acid (Sigma–Aldrich) were used as standards for identification. For the bubble column growth, the total sugar content was measured using the Dubois Method (DuBois et al., 1956).

2.7. 18S rRNA analysis

The genomic DNA was extracted after a grinding procedure using lysing matrix E tubes (6914–500, MP Biomedicals Europe) using a DNeasy Plant Mini kit (Qiagen GmbH, Germany) according to the specifications of the manufacturer. PCR amplification of fragments was done following Kawachi et al. (2012). Forward and reversed primer combination consisted of CV1 × CV2 and CV3 × CV4 primers according to Senousy et al. (2004).

After multiplication, the PCR templates were sequenced with CV1, CV2, CV3 and CV4 sequence primers. Using the PREGAP4 interface of the Staden package 2004 (Staden et al., 2003), raw trace data was processed into assembly ready sequences and sequences were base called by the PHRED base caller (Ewing and Green, 1998). DNA sequence analysis and maximum divergence of 18S rRNA sequences were performed according to the method used by Kawachi et al. (2012).

3. Results

3.1. 18S rRNA analysis

To assess the relationships between our 16 culture collection strains we used 18S rRNA sequence analysis following the method that was used to characterize 31 BOT strains by Kawachi et al. (2012), who strongly linked the *B. braunii* chemical races with their phylogenetic placement. Our strains fit well with Kawachi's results (Fig. 1). Strains UTEX 572, UTEX LB572, AC755, CCALA835, CCALA777, CCALA778, K-1489, CCAP807/2 and SAG30.81 are in subclade 5 which refers to race A. Strains AC759, AC760, AC761 and Showa are in subclade 3 which refers to race B, and strains AC765, AC767 and AC768 in subclade 1 and 2 which refers to race L. In Fig. 2, the assigned subclades 1, 2, 3 and 5 are used to group the strains and therefore it is also included in Tables 2 and 3.

3.2. Shake flask cultivation

The biomass productivity of the 16 strains was determined by assessing the total biomass increase over the cultivation period between time of inoculation and end of experiment (day 18). The biomass productivities in the shake flasks varied up to 2-fold between strains and showed statistically significant differences ($P < 0.05$) in biomass productivity between strains (Fig. 2a). The

Table 4
Volumetric productivity (PV) and maximum specific growth rate (μ_{max}) of *Botryococcus braunii* grown in bubble columns. PV expressed in grams per litre per day and μ_{max} is the amount biomass growth per day.

Strain	PV ($g L^{-1} d^{-1}$)	μ_{max} (d^{-1})
AC755	0.06	0.05
AC759	0.09	0.07
AC761	0.15	0.11
CCALA777	0.08	0.06
CCALA778	0.12	0.17
CCAP807/2	0.14	0.11
Showa	0.14	0.17

Table 5
Percentage of hydrocarbon and total carbohydrate content per biomass dry weight of *Botryococcus braunii* biomass cultured in bubble columns. The measurements were carried out on samples from the last day of growth for each strain.

Strain	% hydrocarbon	% carbohydrates
AC755	16	25
AC759	21	10
AC761	45	10
CCALA777	10	55
CCALA778	0	9
CCAP807/2	7	5
Showa	25	4

three highest biomass producers were AC768, CCALA778 and AC765 with 0.18, 0.15 and 0.14 g L^{-1} day^{-1}, respectively. With 0.08 and 0.07 g L^{-1} day^{-1}, strains AC759 and CCAP807/2 respectively showed the lowest biomass productivity.

The total hydrocarbon content in both the culture medium and biomass was determined at the end of experiment. Long-chain hydrocarbons were detected in seven strains (Fig. 2b). Of these, the highest hydrocarbon producing strains were Showa and AC761, which yielded a hydrocarbon percentage of around 40% per gram biomass dry weight. Strain K-1489 was the lowest producer of the hydrocarbon producing strains, followed by AC755. The strains Showa, AC759, AC760 and AC761 produced C_{33} to C_{37} molecules, whereas K-1984, AC755 and CCAP807/2 produced C_{25} to C_{31} chain hydrocarbons (Table 2). No correlation was found between biomass productivity and hydrocarbon content.

Total carbohydrate content in the culture medium and biomass, based on their monomeric sugar composition, were determined at the end of the growth experiment. Total carbohydrate content varied between 9% and 74% per gram biomass dry weight (Fig. 2c). Two A race strains showed highest amount of total carbohydrates with 74% per biomass dry weight for CCALA777 which is the highest measured, followed by CCALA778 with 52%. The monomeric carbohydrate composition was examined and the main constituent monosugars for the 16 strains are galactose, glucose, fucose, and arabinose (Table 3). Rhamnose was also detected in low but consistent amounts in all strains with values ranging from 2 to 9 mg L^{-1}. The main sugar monomer was galactose for 10 strains, whereas glucose was the main monomer for the remaining six strains studied. Fucose is found to vary significantly between strains, ranging from no detection in AC759, AC760 and AC761 to 463 mg L^{-1} in CCALA778. Other *B. braunii* strains showing high amounts of fucose are AC755, CCALA777 and CCAP807/2 with 172 mg L^{-1}, 279 mg L^{-1} and 136 mg L^{-1} respectively. Of the four race B strains, only Showa showed a small amount of fucose detected with 6 mg L^{-1}. Arabinose varies for most strains between 9 mg L^{-1} and 28 mg L^{-1}, but for Showa it is present in high amounts with 102 mg L^{-1}. Galactose is the largest portion of the total carbohydrate produce in the strains CCALA777 and CCALA778 with 1115 mg L^{-1} and 908 mg L^{-1} respectively. Similarly to hydrocarbon content, no correlation was found between biomass productivity and total carbohydrate.

3.3. Bubble columns cultivation

Based on highest biomass productivity, hydrocarbons and carbohydrate content from the shakeflask experiment, seven strains were scaled-up to bubble columns to assess the cultivation viability. Fig. 3 shows the biomass evolution for these strains grown for up to 30 days in the bubble columns. In Table 4 the volumetric productivities (PV) and maximum specific growth rates (μ_{max}) are given. Growth curves, volumetric productivities and maximum specific growth rates highlight Showa, CCALA778, CCAP807/2 and AC761 as the best performing strains in the bubble columns, with productivities ranging between 0.12 and 0.15 g L^{-1} day^{-1}. The other strains show slower growth with lower biomass productivity. The hydrocarbon and carbohydrate content are shown in Table 5. Long chain hydrocarbons were detected in all seven strains with exception of CCALA778. AC761 shows the highest hydrocarbon content with 45% per gram of biomass dry weight followed by Showa with 25%. The lowest amount of hydrocarbon was found in the strain CCAP807/2 with 7% of its biomass dry weight. Carbohydrate content in the seven strains varied between 4% and 55% for Showa and CCALA777 respectively.

4. Discussion

16 strains of *B. braunii* were compared under identical growth conditions. The results show similar strain variation as observed in the other studies with different growth conditions. And it also shows differences between different studies when comparing the same strains.

In the shakeflask cultivation, biomass productivity varies statistically significantly ($P < 0.05$) among strains in the shake flask cultures, and align with reports from identical strains in similar experimental studies (Eroglu et al., 2011). The strain with the highest productivity (0.18 g L^{-1} day^{-1}) AC768, produced no detectable hydrocarbons and had a relatively low level of carbohydrates (10%, w/w). However, this inverse correlation is not observed for all the strains. For example AC767 and SAG30.81 also show low carbohydrates and no hydrocarbon production, while biomass productivity remained at a low range with 0.10 and 0.07 g L^{-1} day^{-1} respectively. On the other hand strains that produce high amounts of hydrocarbons such as Showa showed much higher productivity (0.11 g L^{-1} day^{-1}). Similar results in the bubble columns were obtained, which suggests that the low biomass productivity of *B.braunii* compared to other algal species is specific and is independent of *B. braunii* producing hydrocarbons or carbohydrates. Low biomass productivity of *B. braunii* at this stage has to be accepted when utilized in large scale production for the extraction of hydrocarbons and carbohydrates until a strain with high productivity can be found that also can produce high amounts of product of interest.

Clear differences are also visible between strains when comparing hydrocarbon content under the same conditions. Seven strains out of sixteen produced hydrocarbons ranging from 6% per gram of biomass dry weight in K-1489 to 42% in the Showa strain, and these values are also found in literature (Metzger and Largeau, 2005). Four strains (Showa, AC759, AC760 and AC761) produce C_{33}– C_{37} hydrocarbons and the remaining three strains for which hydrocarbons were detected (K-1489, AC755, CCAP807/2) produce C_{25}–C_{31} hydrocarbons, in accordance with previous reports (Metzger et al., 1985a; Metzger et al., 1988). For K-1489 strain, this is the first time that the hydrocarbon content is reported. The strain SAG30.81 is reported to contain up to 30% of hydrocarbons (Dayananda et al., 2007) yet in our study no hydrocarbons were detected. Also, for a majority of other strains tested, no hydrocarbons were detected. Media composition between the various previous studies and this work do not vary significantly, therefore the absence of hydro-

carbons in these strains are probably due to other factors. Other cultivation conditions, such as length of cultivation period and light regime might play a role. For example, the growth conditions for AC765, AC767 and AC768 strains were cultivated in cylindrical tubes, aerated with 1% CO_2 under continuous illumination as described (Metzger et al., 1988; Metzger et al., 1985b). For SAG30.81 and UTEX LB572, light conditions were varied with different intensities in continuous and photo-period illumination parameters as described by Dayananda et al. (2007). For CCALA777, the light intensity was of 250 μmol photon m^{-2} s^{-1} in continuous illumination, with 1% CO_2 aeration in cylindrical tubes as described by Fernandes et al. (1991). These differences in the growth conditions could have changed the hydrocarbon synthesis rate and explain why it was not detected in some strains of *B. braunii* in our study. Another possible explanation for these different results might be due to the different methods of hydrocarbon extraction used. In this study we used dichloromethane:methanol as solvent compared to hexane extraction applied by the other studies previously mentioned. But this reason does not stand so well as we have obtained hydrocarbons in the range of 6% to 42% for K-1489 and Showa respectively as well as hydrocarbon chains below and higher than C_{30}.

Differences in total carbohydrate content are also evident ranging from 4% of biomass dry weight in the Showa strain to 74% in the CCALA777 strain. The high viscosity only appears in the CCALA777 and CCALA778. This is not surprising as there are only a few reports in the literature on the ability of *B. braunii* to produce large amounts of carbohydrates (Allard and Casadevall, 1990; Dayananda et al., 2007; Fernandes et al., 1991; Fernandes et al., 1989; Lupi et al., 1994). The highest amount of total carbohydrates extracted from the culture broth in CCALA777 and CCALA778 was 1.78 g L^{-1} and 1.68 g L^{-1} respectively. Similar results are described in literature for the CCALA777 strain, which is also known by its identifier Acoi58 (Fernandes et al., 1989). It is well known the application of carbohydrates in the pharmaceutical industry (Moscovici, 2015) due to the diverse biological active agents present. Carbohydrates from CCALA778 contain 27% of fucose from the total carbohydrates, which translates to 140 g of fucose per Kg of biomass dry weight. Allard and Casadevall (1990) also show similar fucose content in a race A and L strain with 32% of total carbohydrate content. Fucose is considered of high industrial value (Wijesinghe and Jeon, 2012) and it has potential medicinal applications, namely anti-cancer properties (Liao et al., 2013). The carbohydrates of CCALA777 and CCALA778 strains, should be analyzed in depth for its potential applications in industry and as well as more studies to investigate the mechanisms behind the high content of carbohydrates and fucose. Other monomeric composition of the total carbohydrates shows galactose and glucose as the main monomers across all strains. For CCALA777 and CCALA778 galactose is by far the largest fraction of the carbohydrates moieties and is presumably the reason why viscosity increases (Allard and Casadevall, 1990; Díaz Bayona and Garcés, 2014; Fernandes et al., 1989). Recent work shows that galactose can be used for the production of bioethanol via fermentation (Lee et al., 2011; Park et al., 2014), therefore with the high amounts of galactose produced by CCALA777 and CCALA778, these strains can be consider as potential candidates as raw material for the production of bioethanol. Future research in *in situ* extraction of the carbohydrates from these two strains in a continuous cultivation system would further increase the feasibility for commercial scale application.

One point to bear in mind when comparing the strains is the fact that we have non-axenic (bacteria present) strains. Microalgae and bacteria interactions are of major interest as it has been shown that bacteria can have influence on the cultivation of microalgae (Cole, 1982). Therefore we consider that attempts in making *B. braunii* axenic is of advantage as it can improve culture productivities and in this case increase the accuracy of the comparison between strains. Also is of interest to study the possible symbiosis existing with *B. braunii* and bacteria as a recent study using *B. braunii* Ba10 strain shows enhancement of biomass productivities with the presence of bacteria (Tanabe et al., 2015). In this case for example, CCALA778 strain produces high amounts of carbohydrates and if the culture contains high amounts of bacteria, in theory could be that these are degrading the sugars for carbon source and ultimately decreasing the amount of product accumulated and available for harvest.

From the bubble column cultivation, we can only speculate on the results as the batch mode cultivation was run only once for the selected strains. Strains CCALA778, AC761, Showa and CCAP807/2 seem viable for scale up cultivation as volumetric productivities show values in similar range or higher than the shakeflask cultivation. There are differences observed in biomass productivities, such as for AC755 and Showa, and also changes in the carbohydrate and hydrocarbon content. These differences may be explained by physical properties of the cultivation systems used. Biomass productivities can increase in systems such as bubble column reactors, for example because of improved light distribution and availability per cell (Kojima and Zhang, 1999; Ugwu et al., 2005), or better gas mass transfer rates (Posten, 2009). On the other hand, biomass productivities can decrease in these same systems because of shear stress induced by gas purge causing damage to cells (Barbosa et al., 2003) and in case of *B. braunii*, possibly damage to the colonies. Further work with strains such as CCALA778 and AC761 should be done at larger scale to optimize and characterize their potential as biofuel and bioased raw materials.

Two aspects for future research and comparison of strains are (1) the characterization of the extracellular and intracellular hydrocarbons. Because *in situ* ("milking") extraction is a viable option as shown by Moheimani et al. (2013), a wide comparison would inform also which strains are best for hydrocarbon production facility. (2) the characterization of colony formation and structure as it could affect the extractability of products, growth rates related to light absorption and possibly downstream processes.

What also has been shown and is again evident, is the variability of the physiological characteristics of *B. braunii* strains relative to different literature studies. Therefore there would be a benefit of improving the species reference or taxonomy catalogue when dealing with the cosmopolitan *B. braunii*, for example, a similar approach taken by Darienko et al. (2015), who described the closely related species Coccomyxa using integrative taxonomy and DNA barcoding. This type of approach is suitable for microalgae that have high morphological and physiological similarities. A recent publication by Hegedűs et al. (2016) follows similar approach with *B. braunii* A races.

5. Conclusion

This study presents the physiological diversity of commercially available *B. braunii* strains grown in similar conditions with respect to biomass productivity, and hydrocarbon and carbohydrate content. The physiological characteristics of *B. braunii* from this study complemented with the literature can be as diverse as the number of strains compared. This variability keeps scientists challenged in the characterization and understanding of *B. braunii* and at the same time positive that the exploration of this specie can in the future yield a strain that will have the minimum required characteristics for industrial application. CCALA778 strains show potential as carbohydrate producers with feasible applications in different industries. For example, galactose in fermentation processes for the production of bioethanol and fucose for the application in cancer treatment. For hydrocarbons production the strain AC761 could be exploited. The slow growth of *B. braunii* is not correlated to

the hydrocarbon content or total carbohydrate, but it remains the main obstacle to overcome for large scale production, this could also be overcome if applications with high value can emerge. For future work more can be done on the characterization of extracellular and intracellular hydrocarbon in the different strains, as well colony structure, as these will have an impact on the extractability of hydrocarbons and carbohydrates.

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